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RESEARCH PAPER

Anti-inflammatory effects of selective glucocorticoid receptor modulators are partially dependent on up-regulation of dual specificity phosphatase 1

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BACKGROUND AND PURPOSE

It is thought that the anti-inflammatory effects of glucocorticoids (GCs) are largely due to GC receptor (GR)-mediated transrepression of NF-κB and other transcription factors, whereas side effects are caused by activation of gene expression (transactivation). Selective GR modulators (SGRMs) that preferentially promote transrepression should retain anti-inflammatory properties whilst causing fewer side effects. Contradicting this model, we found that anti-inflammatory effects of the classical GC dexamethasone were partly dependent on transactivation of the dual specificity phosphatase 1 (DUSP1) gene. We wished to determine whether anti-inflammatory effects of SGRMs are also mediated by DUSP1.

EXPERIMENTAL APPROACH

Dissociated properties of two SGRMs were confirmed using GR- and NF-κB-dependent reporters, and capacity to activate GC-responsive elements of the DUSP1 gene was tested. Effects of SGRMs on the expression of DUSP1 and pro-inflammatory gene products were assessed in various cell lines and in primary murine *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$macrophages.

KEY RESULTS

The SGRMs were able to up-regulate DUSP1 in several cell types, and this response correlated with the ability of the compounds to suppress COX-2 expression. Several anti-inflammatory effects of SGRMs were ablated or significantly impaired in *Dusp1*$^{-/-}$ macrophages.

CONCLUSIONS AND IMPLICATIONS

Like dexamethasone, SGRMs appear to exert anti-inflammatory effects partly via the up-regulation of DUSP1. This finding has implications for how potentially therapeutic novel GR ligands are identified and assessed.

Abbreviations

AP-1, activating protein 1; BMM, bone marrow-derived macrophage; CXCL1, chemokine (CXC motif) ligand 1; dex, dexamethasone; DUSP1, dual specificity phosphatase 1; GC, glucocorticoid; GR, GC receptor; GRE, GC response element; GRR, GC responsive region; SEGRA, selective GR agonist; SGRM, selective GR modulator

Introduction

For more than half a century, synthetic glucocorticoids (GCs) have been extensively used to treat chronic inflammatory diseases such as rheumatoid arthritis, asthma and inflammatory bowel diseases (Barnes, 2006; Hillier, 2007). The basis of their therapeutic action is the impairment, in most cell types, of the expression of pro-inflammatory genes. Various unpredictable and occasionally life-threatening side effects of GCs have been documented since their earliest clinical use (Schacke *et al.*, 2002). These include osteoporosis and diabetes mellitus, atrophy of skin and muscle, hypertension and increased susceptibility to infection. Nevertheless, GCs are still the cornerstone of treatment for many diseases. At the same time, major research initiatives attempt to separate the desired anti-inflammatory effects of GCs from their side effects (Schacke *et al.*, 2007; Hudson *et al.*, 2008; De Bosscher *et al.*, 2010).

GCs modulate gene expression via the GC receptor (GR), a transcription factor belonging to the nuclear hormone receptor superfamily (Newton, 2000; Tuckermann *et al.*, 2005). Lipophilic ligands such as the endogenous GC cortisol or the synthetic GC dexamethasone (dex) diffuse across the cell membrane and bind to GR in the cytoplasm. This promotes the release of GR from a large complex of chaperone proteins and its migration to the nucleus. In most but not all cases, transcriptional activation by GR (transactivation) is dependent on homodimerization, which is mediated by a short motif adjacent to the first of two zinc finger DNA-binding motifs. GR homodimers recognize sequences related to the idealized, palindromic consensus AGAACAnnnTGTTCT (GC response element or GRE).

A second physiologically important function of GR is to inhibit transcription via a mechanism known as transrepression (Kassel and Herrlich, 2007; De Bosscher *et al.*, 2010; Glass and Saijo, 2010). In this case, GR does not bind directly to DNA but instead is recruited to DNA via direct or indirect interactions with other transcription factors, notably members of the activating protein 1 (AP-1) and NF-κB families, both of which play important roles in the expression of pro-inflammatory genes. The presence of GR at AP-1 or NF-κB binding sites is thought to inhibit transcriptional activation by impairing recruitment of transcriptional co-activators, or by promoting recruitment of co-repressors.

It is often stated that the anti-inflammatory effects of GCs are largely mediated by transrepression, whereas side effects are largely mediated by transactivation. If this is correct, it may be possible to improve upon classical GCs by identifying novel ligands of GR that selectively promote its transrepressive function rather than its transactivating function (Schacke *et al.*, 2007; Berlin, 2010; De Bosscher *et al.*, 2010; Newton *et al.*, 2010). Such compounds are known as dissociated GR ligands, selective GR agonists (SEGRAs) or modulators (SGRMs). They are predicted to retain anti-inflammatory effects of classical GCs like dex but cause fewer or less severe side effects. Typically, SGRMs have been identified from drug libraries first on the basis of affinity for GR and second on the basis of effects on reporter constructs. Transactivation is tested against well-known GC target genes such as tyrosine aminotransferase (TAT), or against constructs that contain well-characterized GC responsive promoters or multimerized GR binding sites. Transrepression is tested using promoters that contain AP-1 and/or NF-κB binding sites and are activated by pro-inflammatory stimuli. Alternatively, reporters containing multimerized AP-1 or NF-κB binding sites may be used. As reviewed elsewhere (Schacke *et al.*, 2007; Berlin, 2010; De Bosscher *et al.*, 2010), a number of interesting compounds have been identified using this basic approach. Recently described examples include ZK216348 and LGD-552, which are non-steroidal GR ligands (Schacke *et al.*, 2004; Humphrey *et al.*, 2006; Miner *et al.*, 2007; Lopez *et al.*, 2008).

As well as directly inhibiting expression of pro-inflammatory genes by means of transrepression, GCs can exert indirect therapeutic effects, via the up-regulation of several anti-inflammatory genes (Clark, 2007; Newton and Holden, 2007). For example, many cell types respond to GCs by expressing dual specificity phosphatase (DUSP1), an enzyme that dephosphorylates and inactivates both p38 MAPK and JNK (Abraham and Clark, 2006; Owens and Keyse, 2007). The up-regulation of DUSP1 has been suggested to contribute to destabilization of pro-inflammatory mRNAs (Lasa *et al.*, 2001; 2002; Quante *et al.*, 2008) and to the inhibition of AP-1 and NF-κB function (Diefenbacher *et al.*, 2008; Bladh *et al.*, 2009; Cho and Kim, 2009; King *et al.*, 2009). Correspondingly, many of the anti-inflammatory effects of GCs are impaired in macrophages derived from *Dusp1*$^{-/-}$ mice, or cells in which DUSP1 has been down-regulated using RNA interference (Abraham *et al.*, 2006; Furst *et al.*, 2007; Issa *et al.*, 2007; Kang *et al.*, 2008; Quante *et al.*, 2008; King *et al.*, 2009). *In vivo* anti-inflammatory effects of dex were dependent on DUSP1 in experimental models of acute inflammation (Abraham *et al.* 2006; Wang *et al.*, 2008), asthma (Li *et al.*, 2010) and rheumatoid arthritis (our unpublished results). There are therefore problems with a paradigm that equates anti-inflammatory effects of GCs with transrepression and not transactivation. In fact, such a model is not strongly supported by experimental evidence. For example, there is no genetically modified mouse strain that clearly demonstrates a separation between side effects and transactivation on one hand, and transrepression and anti-inflammatory effects on the other. A knock-in mouse strain expressing a dimerization defective mutant of GR (known as GR^{dim}) was initially thought to provide evidence of just such a mechanistic separation between therapeutic and harmful effects (Tuckermann *et al.*, 1999), but emerging complexities in the phenotype of the GR^{dim} mouse now undermine rather than support the paradigm (Kleiman and Tuckermann, 2007; Frijters *et al.*, 2010; Rauch *et al.*, 2010). Recent results also show that, although SGRMs were identified on the basis of impaired transcriptional activation, they may be quite capable of inducing expression of DUSP1 and other genes with anti-inflammatory roles (Chivers *et al.*, 2006; Janka-Junttila *et al.*, 2006; Lopez *et al.*, 2008; Newton *et al.*, 2010). We therefore asked whether anti-inflammatory effects of SGRMs may actually be dependent on the induction of DUSP1. The answer to this question will have an important impact on how novel GR ligands with improved therapeutic indices are discovered, and how their properties are to be understood.

Methods

Mice and reagents

All animal procedures were performed under United Kingdom Home Office regulations and with local Ethical Review Committee approval. *Dusp1*$^{-/-}$ mice were generated as described previously (Dorfman *et al.*, 1996). They were back-crossed against C57BL/6 for 10 generations to generate a colony with almost pure (99.9%) C57BL/6 genetic background, genotype at the *Dusp1* locus being determined by a PCR assay. The same breeding programme was used to generate a *Dusp1*$^{+/+}$ colony with equivalent genetic background. Age- and sex-matched *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ animals were used to generate bone marrow-derived macrophages (BMMs) by differentiation from BM haematopoietic stem cells for 5 days in RPMI 1640 medium supplemented with 10% FCS, 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin (PAA) and 10 ng·mL^{-1} M-CSF (Peprotech, London, UK). This method routinely generates macrophages of at least 85% purity (Lari *et al.*, 2007). Dulbecco's modified Eagle's medium (DMEM, PAA) supplemented with 10% FCS was used for the culture of the HeLa, RAW264.7 and A549 cell lines. Stably transfected A549 cells were maintained with additional 0.2 mg·mL^{-1} of G-418 (Peprotech). All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C and seeded to confluence (unless otherwise stated) in FCS supplemented medium the day before the experiment. Cells were treated with different doses of dex (Sigma, Dorset, UK), Cpd1 and Cpd2 (Roche, Palo Alto, CA, USA) or vehicle [dimethyl sulfoxide (DMSO) Sigma] and stimulated with 1 ng·mL^{-1} IL-1β (made in house) or 10 ng·mL^{-1} LPS (Alexis Biochemicals, Exeter, UK).

Plasmids, transfection and luciferase assays

The GRE A549 reporter line (pGL3.neo.TATA.2GRE) was a gift from R Newton (University of Calgary). The NF-κB-dependent reporter containing three κB binding sites linked to a TATA box and a firefly luciferase coding sequence (3κBtkluc) was also generously provided by R Newton. pGL3b-Hs.-4.8, pGL3p-Mm-29GRR, -Hs-1.3GRR and -Hs-4.6GRR were as previously described (Tchen *et al.*, 2010). Cells were seeded to approximately 50% confluence the day before the experiment then transiently transfected using Superfect (Qiagen, Crawley, UK) with 200 ng of *firefly* luciferase reporters as indicated plus 100 ng of *Renilla* luciferase expression vector and pBluescript (Agilent Technologies, Edinburgh, UK) as carrier to make the total quantity of DNA up to 1 μg. Following transfection, cells were treated with vehicle (0.1% DMSO), Dex, Cpd1 or Cpd2 as described. Cells were harvested, and luciferase activities were measured using the dual luciferase reporter assay kit (Promega, Southampton, UK) and Microbeta luminometer (PerkinElmer Life Sciences, Seer Green, UK). Firefly luciferase activities were normalized against *Renilla* luciferase activities.

Western blotting

Whole cell lysates were harvested in ice-cold lysis buffer [50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 1% Triton X-100, 10% glycerol, 0.5% NP40, 10 mM NaF, 1 mM sodium orthovanadate, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride, 3 μg·mL^{-1} aprotinin, 23 μM E64]. Lysates were clarified by centrifugation at 13 000× *g* for 1 min at 4°C, and protein concentrations were measured using Bradford assay. Equal amounts of total protein were loaded on SDS-PAGE gels. After electrophoresis samples were transferred to PVDF membranes (PerkinElmer Life Science), probed with anti-DUSP1, anti-COX-2 and anti-tubulin primary antibodies (Santa Cruz, Heidelberg, Germany; Cayman Chemical, Tallinn, Estonia and Sigma, respectively) then with appropriate horseradish peroxidase-coupled secondary antibodies (Dako, Cambridge, UK). Proteins were detected using the enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK). COX-2 protein expression was estimated by scanning densitometry of Western blots using a calibrated imaging densitomer (GS-710; Bio-Rad Laboratories, Hertfordshire, UK) and the Phoretix ID software.

Measurement of cytokine expression

Supernatants were collected and stored at –20°C until used for measurement of cytokine proteins. IL-12p40 protein was detected by ELISA, using an eBioscience kit, according to the manufacturer's instructions. IL-6, TNF-α and CXCL-1 proteins were detected simultaneously using the xMap Technology from Luminex® and 96-well filter plates (Millipore, Dundee, UK). Briefly, colour-coded Bio-plex beads (Bio-Rad Laboratories), or microspheres, were coupled to antibodies against IL-6, TNF-α and CXCL-1 (R&D Systems, Abingdon, UK) using an amine coupling kit from Biacore Life Sciences (Buckinghamshire, UK). Beads were coated with 50 μg·mL^{-1} of the primary antibodies; standards and samples were then added and left overnight at 4°C. After addition of 0.5 μg·mL^{-1} of the corresponding biotinylated secondary antibodies and streptavidin-PE (Peprotech), cytokines were detected by laser excitation of each internal dye identifying the different microspheres, using the Luminex® 100 Total System.

Measurement of mRNAs

Total cellular RNA was isolated using the RNAeasy® Mini Kit (QIAGEN, Crawley, UK), performing the recommended on-column DNase treatment step. mRNA levels were measured by quantitative real-time PCR using One-Step RT qPCR Mix from Eurogentec (Seraing, Belgium) and TaqMan probes purchased from Applied Biosystems (Paisley, UK) (mouse GAPDH Mm99999915_g1, DUSP1 Mm00457274_g1, COX2 Mm00478374_m1, human GAPDH Hs99999905_g1, DUSP1 Hs00610256_g1, COX-2 Hs00153133_m1). The PCRs were performed in a total volume of 10 μL. The amplification condition consists of an initial reverse transcriptase step of 30 min at 48°C, and 10 min denaturation at 95°C followed by 45 cycles of 3 s at 95°C and 30 s at 60°C. Rotor-Gene 3000 (Corbett Research Ltd, Crawley, UK) was used to quantify the mRNAs. Changes in abundance were assessed by the comparative threshold cycle (ΔCt) method and normalized against GAPDH (measured by the same method).

Statistics and calculation of dissociation indices

Statistical analysis was performed using one-sample *t*-test or ANOVA with the Bonferroni's post-test for multiple comparisons. All tests were performed using Prism software version 5 (GraphPad, La Jolla, CA, USA). A P-value <0.05 was considered significant.

Dissociation indices were calculated for Cpd1 and Cpd2, based on their potencies, in other words, the EC_{50} values for activation of a GRE-dependent reporter and inhibition of an NF-κB-dependent reporter, relative to the corresponding values for dex.

$$\text{Dissociation index} = [EC_{50}{}^{\text{transactivation}}\ \text{Cpd}/EC_{50}{}^{\text{transrepression}}\ \text{Cpd}] \times [EC_{50}{}^{\text{transrepression}}\ \text{dex}/EC_{50}{}^{\text{transactivation}}\ \text{dex}].$$

Dissociation indices were also calculated on the basis of the efficacies of Cpd1 and Cpd2 for activation of the GRE-dependent reporter and repression of the NF-κB-dependent reporter, relative to the corresponding values for dex.

$$\text{Dissociation index} = [\text{Fold activation}^{\text{GRE}}\ \text{Cpd}/\text{Fold inhibition}^{\text{NF-κB}}\ \text{Cpd}] \times [\text{Fold inhibition}^{\text{NF-κB}}\ \text{dex}/\text{Fold activation}^{\text{GRE}}\ \text{dex}].$$

Results

Compounds

The two compounds used in this study were selected from patents registered by two companies that have led research into SGRMs. Compound 1 (Jaroch *et al.*, 2002) is closely related to ZK 216348 (Schacke *et al.*, 2004) and ZK 245186 (Schacke *et al.*, 2009) (Figure 1). In THP-1 cells stimulated with LPS, Cpd1 inhibited the expression of IL-8 with 77% efficacy and EC_{50} of 4.3×10^{-9} M. For comparison, prednisolone (pred) inhibited expression of IL-8 with 95% efficacy and EC_{50} of 2.4×10^{-8} M. In the croton oil ear oedema model, pred and Cpd1 were similarly effective at a dose of 30 mg·kg^{-1}, inhibiting inflammation by 81% and 84%. In the same model, pred induced liver tyrsoine aminotransferase (a surrogate marker of metabolic side effects) by 8-fold, Cpd1 by 3.7-fold (Jaroch *et al.*, 2002). Cpd2 (Coghlan *et al.*, 1999; Kym *et al.*, 2003) is very closely related to LGD-5552 (Lopez *et al.*, 2008) (Figure 1). It is highly selective for GR over progesterone receptor (PR), the respective K_i values being 1.5×10^{-9} M and 1.434×10^{-6} M.

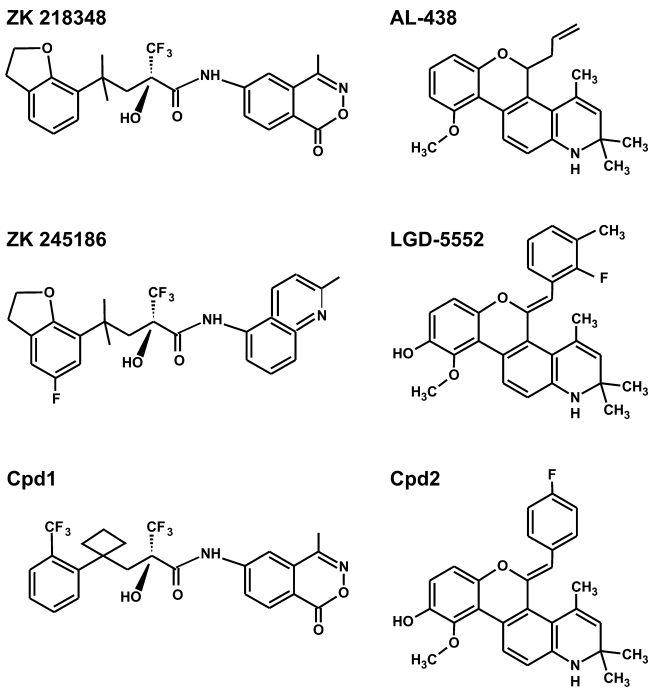

Figure 1
Molecular structures of Cpd1, Cpd2 and near relatives.

Dissociated properties of Cpd1 and Cpd2

Dissociated properties of Cpd1 and Cpd2 were determined using reporters with multimerized binding sites for GR and NF-κB. Similar methods have previously been used to identify SGRMs (Vayssiere *et al.*, 1997) and continue to be used as screening tools in the pharmaceutical industry. The transactivation functions of Cpd1 and Cpd2 were assessed using an A549 pulmonary epithelial cell line stably transfected with a luciferase reporter containing two tandem GREs derived from the rat TAT gene upstream of a minimal TATA box from the rabbit β-globin gene (Figure 2A) (Chivers *et al.*, 2006). Dex activated this construct with A_{max} of 16.4 and EC_{50} of 4.1×10^{-9} M. The novel GR ligands up-regulated luciferase expression with A_{max} and EC_{50} values of 8.4 and 2.9×10^{-8} M (Cpd1); 5.9 and 1.30×10^{-7} M (Cpd2), respectively. Both Cpd1 and Cpd2 therefore have relatively low efficacy and potency in this assay of transcriptional activation. Activation of the GRE reporter by dex, Cpd1 or Cpd2 was effectively blocked by an equivalent concentration of the GR antagonist RU486 (mifepristone), confirming that the SGRMs regulate transcription via GR (Figure 2C). Since Cpd1 and Cpd2 appeared to function as partial agonists of GR-mediated transcription, we considered whether they might impair transcriptional activation by a full agonist such as dex. To address this question, the GRE reporter was activated by 10^{-8} M dex in the presence of increasing concentrations of Cpd1 or Cpd2, from 10^{-9} to 10^{-7} M (Figure 2D). Statistically significant impairment of dex-induced transcription was observed only in the presence of a 10-fold molar excess of Cpd2 over dex.

Transrepression was then tested following transient transfection of A549 cells with a luciferase reporter construct containing three NF-κB binding sites from the human COX-2 gene upstream of a minimal TATA box (Figure 2B) (Holden *et al.*, 2007). This construct was strongly activated by IL-1β and dose-dependently inhibited by dex with an EC_{50} of 3.4×10^{-9} M. However, the extent of inhibition did not exceed 67%. Cpd1 and Cpd2 impaired the activation of the NF-κB reporter with similar efficacy (53% and 54%) and potency (EC_{50} values of 7.8×10^{-9} M and 3.5×10^{-9} M). Dissociation indices were calculated for Cpd1 and Cpd2, based on their potency and efficacy of transactivation and transrepression, relative to the corresponding values for the reference compound dex (see Methods). In either case, a dissociation index greater than 1 indicates selective capacity for transrepression over transactivation. By potency, Cpd1 and Cpd2 had dissociation indices of 3.04 and 30.8, respectively. By efficacy, their dissociation indices were 2.53 and 4.32, respectively. Both compounds therefore appeared to conform to the description of selective GR modulators.

The extent of inhibition of a NF-κB reporter by both dex and the SGRMs was less than anticipated; for example, it was

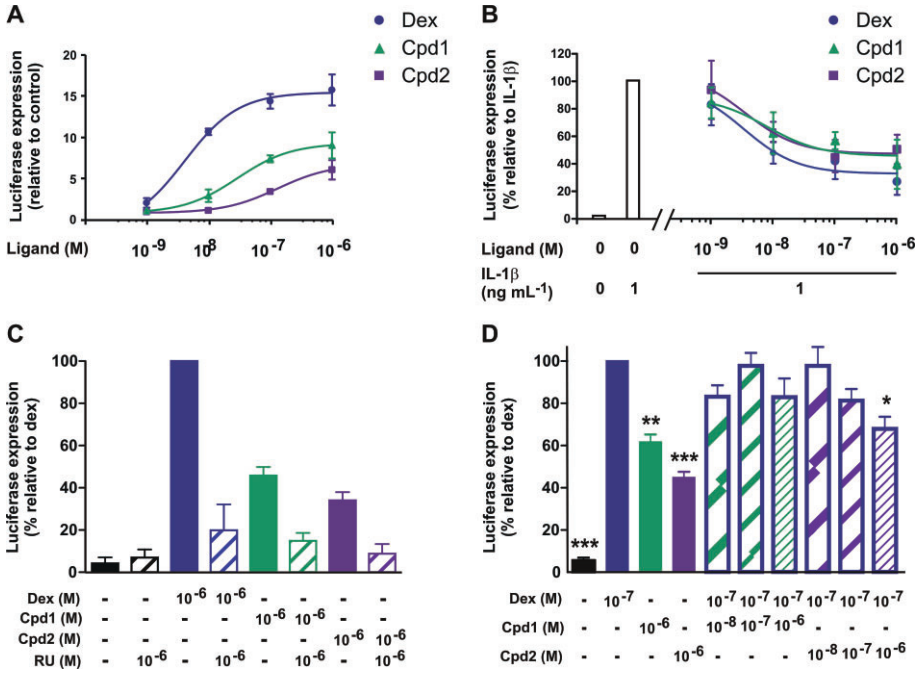

Figure 2

Dissociation parameters and GR dependence of Cpd1 and Cpd2. (A) A549 cells stably transfected with a GRE-dependent reporter were incubated for 6 h with vehicle (0.1% DMSO) or increasing concentrations (10^{-9} to 10^{-6} M) of dex, Cpd1 and Cpd2. Cell lysates were prepared, and luciferase activities were measured. Graph indicates mean ± SEM luciferase activities relative to vehicle treated control cells from four independent experiments. (B) A549 cells were transiently transfected with a reporter plasmid expressing firefly luciferase under the control of three tandem NF-κB sites, and with a control plasmid expressing *Renilla* luciferase. The following day, cells were pretreated for 2 h with increasing concentrations (10^{-9} to 10^{-6} M) of dex, Cpd1 and Cpd2, then stimulated with 1 ng·mL^{-1} of IL-1β for 6 h. Cell lysates were harvested, and firefly and *Renilla* luciferase activities were measured. Firefly luciferase activities were normalized against *Renilla* luciferase activities to correct for variations in transfection efficiency. The graph represents % of the activity of the IL-1β-stimulated cells ± SEM from four independent experiments. (C) The A549 GRE reporter cell line was incubated for 6 h with vehicle (0.1% DMSO) or 10^{-6} M dex, Cpd1 or Cpd2 in the absence or presence of 10^{-6} M RU486 (RU). Luciferase activities are expressed relative to that of cells treated with dex alone. The graph shows averages from two independent experiments ± SD. (D) The A549 GRE reporter cell line was incubated for 6 h with vehicle, dex and/or SGRMs at the concentrations indicated. Luciferase activities are expressed relative to that of cells treated with 10^{-7} M dex alone. The graph shows averages from three independent experiments ± SEM. ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$ relative to cells treated with 10^{-7} M dex alone.

invariably less than the extent of down-regulation of COX-2 mRNA in the same cells (see later, Figure 5). We tested responses of the same NF-κB reporter in a stably transfected A549 cell line; used an alternative reporter based on NF-κB binding sites from the HIV long terminal repeat; or varied concentrations of IL-1β between 0.2 and 10 ng·mL^{-1}. Under no condition was reporter gene expression inhibited by more than 70% (data not shown).

SGRMs are able to induce expression of DUSP1

We and others previously identified two GC responsive regions (GRRs) within the human DUSP1 5′ region, at –1.3 and –4.6 kb with respect to the transcription start site (Johansson-Haque *et al.*, 2008; Shipp *et al.*, 2010; Tchen *et al.*, 2010). Activation of GRR-4.6 by dex was impaired by mutation of the dimerization domain of GR, whereas activation of GRR-1.3 by dex was insensitive to this mutation (Tchen *et al.*, 2010), suggesting that GR may interact differently with the two sites. Having established that SGRMs were capable of transcriptional activation of a stereotypical GRE reporter, we asked whether they could also regulate transcription via GRR-1.3 and -4.6 (Figure 3). The reporter constructs pGL3p-GRR-1.3 and pGL3p-GRR-4.6 (Tchen *et al.*, 2010) are based on the pGL3p vector (Promega), in which firefly luciferase is expressed under the control of an SV40 early promoter. Upstream of the SV40 promoter, pGL3p-GRR-1.3 contains the region of the human Dusp1 locus from –1366 to –1237 with respect to the transcription start site. pGL3p-GRR-4.6 contains the region –4834 to –4369. Cpd1 and Cpd2 significantly activated transcription via both GRR-1.3 and GRR-4.6. The proximal element (GRR-1.3) was slightly less responsive to the two SGRMs than to dex. The distal element (GRR-4.6) was approximately five-fold more responsive to dex than to either of the SGRMs. It was also established by chromatin IP (ChIP), that all three ligands could promote recruitment of GR to both GRR-1.3 and GRR-4.6 in HeLa cells (not shown).

The ability of GR ligands to induce expression of DUSP1 mRNA and protein was next tested in A549, HeLa (human epithelial carcinoma cell line), RAW264.7 (mouse macrophage-like cell line) and BMM (primary mouse bone marrow-derived macrophages) (Figure 4). Note that BMMs displayed anomalous responses to the highest doses of GR

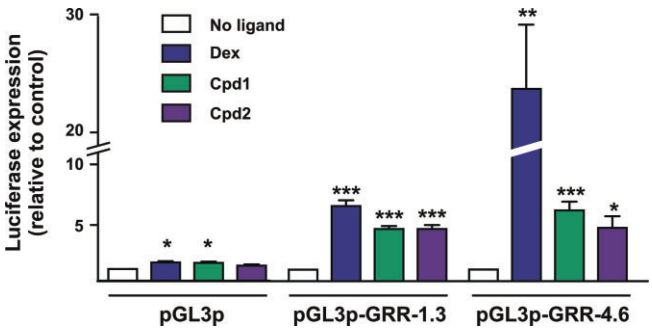

Figure 3

Cpd1 and Cpd2 activate transcription via GC-responsive regions of the human DUSP1 gene. HeLa cells were transiently transfected with pGL3p or derivatives that contain GRR-1.3 or GRR-4.6 from the human DUSP1 gene. A plasmid expressing *Renilla* luciferase was co-transfected as a control for transfection efficiency. Cells were treated with vehicle (0.1% DMSO) or 10^{-7} M GR ligands as indicated for 6 h, then lysates were prepared, firefly and *Renilla* luciferase activities were measured and fold responses to ligand were calculated. The graph represents mean ± SEM from three (pGL3p), six (pGL3p-GRR-1.3) or seven (pGL3p-GRR-4.6) independent experiments. ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$ relative to vehicle-treated cells.

ligands (Figure 4D), possibly due to effects on cell viability or proliferation. This phenomenon was not investigated further. The pattern of response of the DUSP1 gene was highly variable between cell types and was not predictable from the response of the GRE reporter, even within a single cell type. For example, in A549 cells Cpd2 was a poor activator of the GRE reporter but activated the endogenous DUSP1 gene with similar efficacy to dex (although with a rather higher EC_{50}). In HeLa cells, the endogenous DUSP1 gene responded similarly to dex and Cpd2. Cpd1 reached the same efficacy (fold activation) but had a higher EC_{50} (lower potency) than the other two compounds. Compared to dex, both SGRMs had low efficacy and potency in RAW264.7 cells and in primary murine macrophages. Rank orders of efficacy and potency of the three compounds for activation of DUSP1 gene expression were not the same for any two cell lines, illustrating the extreme variability of response.

Anti-inflammatory effects of SGRMs are partially dependent on DUSP1

Having shown that SGRMs are able to induce expression of DUSP1, we investigated the relationship between DUSP1 up-regulation and anti-inflammatory effects of SGRMs in A549 cells (Figure 5) RAW264.7 cells and mouse macrophages (Figure 6). Suppression of COX-2 was selected as a read-out of anti-inflammatory efficacy, because this gene is up-regulated by different pro-inflammatory stimuli in many cell types, and is a well characterized GC target with NF-κB sites in its promoter. In A549 cells, the down-regulation of Cox-2 mRNA and protein mirrored the up-regulation of Dusp1 mRNA and protein (Figure 5A–C). Inhibition of COX-2 protein expression was estimated by scanning densitometry of Western blots. EC_{50} values for induction of Dusp1 mRNA and inhibition of COX-2 mRNA and COX-2 protein were calculated. EC_{50} values

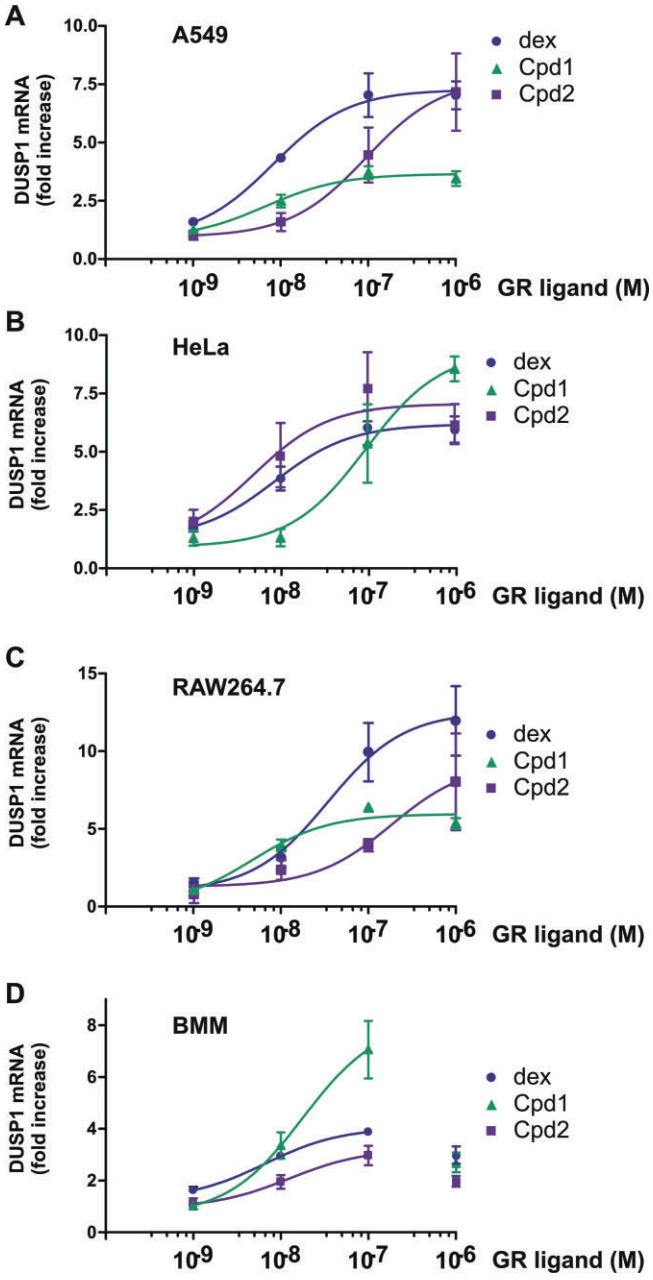

Figure 4

Induction of DUSP1 mRNA by Cpd1 and Cpd2. (A) A549 (B) HeLa (C) RAW cells and (D) BMM were treated with increasing concentrations (10^{-9} to 10^{-6} M) of dex, Cpd1 and Cpd2 for 1 h. mRNA was harvested and DUSP1 expression quantified using RT-PCR. For each cell type, results were presented as fold induction relative to cells treated with vehicle alone (0.1% DMSO). Graphs indicate means ± SEM from at least three independent experiments.

for induction of DUSP1 protein could not be determined because of non-specific background in the Western blots. For each GR ligand, the calculated EC_{50} values for DUSP1 induction and COX-2 inhibition were close to one another (Table 1). At the mRNA level, the correspondence between Dusp1 up-regulation and Cox-2 inhibition can be seen in the symmetry of the dose-response curves (Figure 5C). RU486 incom-

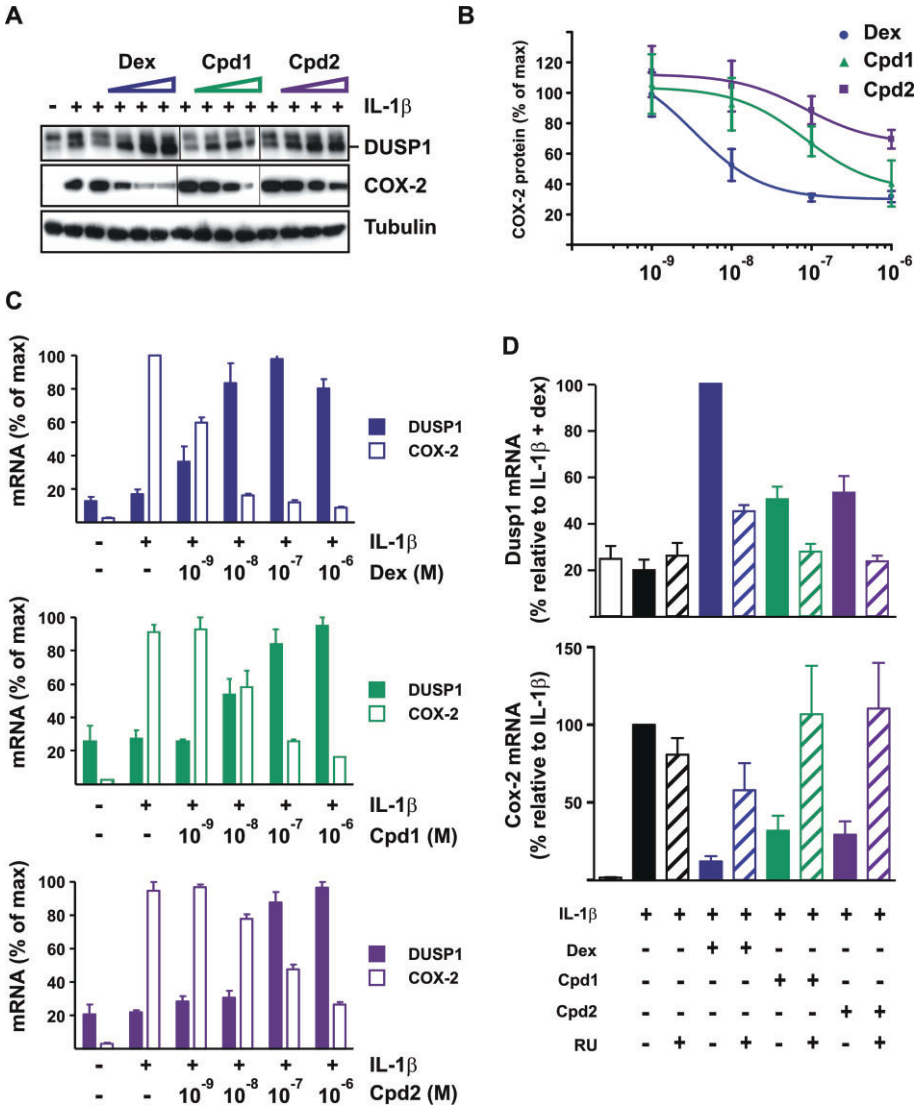

Figure 5

Effects of Cpd1 and Cpd2 on the expression of DUSP1 and COX-2 in A549 cells. (A, B, C) A549 cells were pretreated for 2 h with vehicle (0.1% DMSO) or increasing concentrations (10^{-9} to 10^{-6} M) of dex, Cpd1 or Cpd2 then challenged for 4 h with 1 ng·mL^{-1} of IL-1β. (A) Representative Western blot of DUSP1 and COX-2 protein expression. (B) COX-2 protein expression was quantified by scanning densitometry in three independent experiments. (C) Dusp1 and Cox-2 mRNA were measured by real-time PCR and plotted relative to maximum expression. Graphs show averages ± SEM from three independent experiments. (D) A549 cells were treated with IL-1β (1 ng·mL^{-1}), RU486 (RU), dex, Cpd1, Cpd2 (each 10^{-6} M) in the combinations indicated. Dusp1 and Cox-2 mRNAs were measured and expressed with respect to cells treated with either IL-1β alone (COX-2) or IL-1β + dex (Dusp1). Graphs represent averages ± SEM from four independent experiments.

pletely blocked the up-regulation of Dusp1 mRNA by dex and incompletely rescued COX-2 from dex-mediated suppression (Figure 5D). RU486 completely prevented the up-regulation of Dusp1 mRNA by Cpd1 or Cpd2 and effectively rescued COX-2 mRNA from inhibition by either of the SGRMs. Like dex, Cpd1 and Cpd2 therefore exert anti-inflammatory effects via GR and not via an off-target mechanism.

In RAW264.7 cells (Figure 6A) or primary mouse macrophages (Figure 6B) stimulated with LPS, the up-regulation of *Dusp1* and down-regulation of *COX-2* mRNAs by GR ligands displayed similar dose-dependence, again demonstrated by the symmetry of the dose-response curves. In BMM, Cpd1 and Cpd2 up-regulated *Dusp1* expression with similar A_{max} (5 and 5.4, respectively), slightly less than the A_{max} value of 7.1 for induction of *Dusp1* by dex. Cpd1 induced *Dusp1* with EC_{50} of 1.4×10^{-9} M; therefore, *Dusp1* expression was near maximal at both 10^{-8} and 10^{-6} M. Cpd2 induced *Dusp1* with a considerably higher EC_{50} of 1.9×10^{-8} M; therefore, expression was still increasing in the range 10^{-8} and 10^{-6} M. These observations provide context for the following analysis of responses to SGRMs in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages.

The above results are consistent with, but do not prove, an important role for DUSP1 in the anti-inflammatory effects

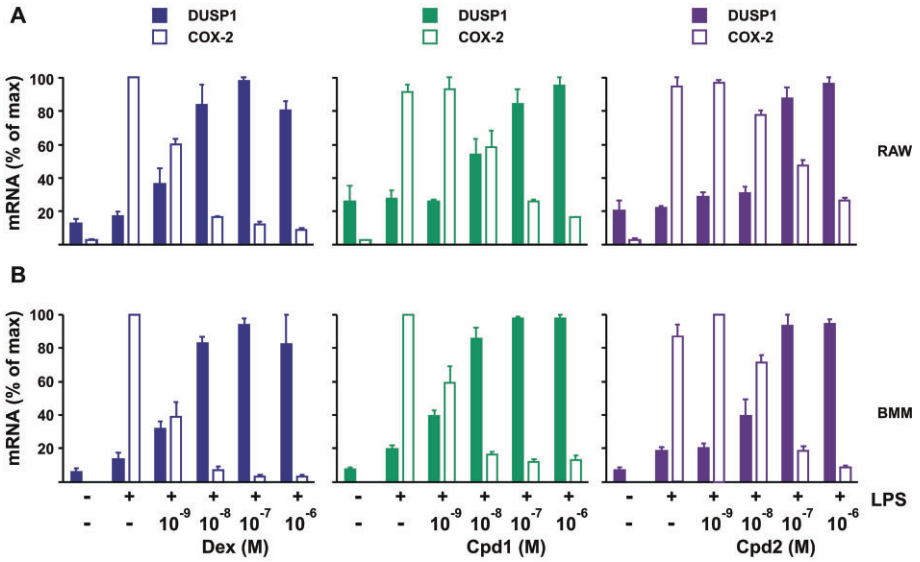

Figure 6

Effects of Cpd1 and Cpd2 on the expression of DUSP1 and COX-2 in RAW264.7 cells and BMMs. RAW264.7 cells (A) or BMMs (B) were pretreated for 2 h with vehicle (0.1% DMSO) or increasing concentrations (10^{-9} to 10^{-6} M) of dex, Cpd1 or Cpd2 then challenged for 4 h with 10 ng·mL^{-1} of LPS. *COX-2* and *Dusp1* mRNAs were measured and plotted as in Figure 5D.

Table 1

EC_{50} values for induction of DUSP1 mRNA, inhibition of COX-2 mRNA and protein expression in A549 cells

	EC_{50} (M) DUSP1 mRNA	COX-2 mRNA	COX-2 protein
Dex	7.0×10^{-9}	6.3×10^{-9}	5.2×10^{-9}
Cpd 1	1.52×10^{-8}	1.01×10^{-8}	3.35×10^{-8}
Cpd 2	3.83×10^{-8}	3.19×10^{-8}	8.52×10^{-8}

Values were calculated on the basis of the data illustrated in Figure 5B and C.

of both dex and SGRMs. To investigate this further, we tested effects of GR ligands on the expression of several pro-inflammatory genes in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ mouse BMMs. As previously reported (Chivers *et al.*, 2006; Hammer *et al.*, 2006), *Dusp1*$^{-/-}$ macrophages overexpressed IL-6 and TNFα proteins when stimulated with LPS, whilst the expression of IL-12p40 was significantly less than in *Dusp1*$^{+/+}$ macrophages (Figure 7). Increased expression of *COX-2* mRNA in the knockout macrophages did not reach statistical significance. In agreement with previous observations (Abraham *et al.*, 2006), 10^{-8} or 10^{-6} M dex strongly decreased the expression of TNF protein in *Dusp1*$^{+/+}$ macrophages but had relatively little effect in *Dusp1*$^{-/-}$ macrophages (Figure 8). We previously reported that dex down-regulated *Il-6* mRNA in a manner largely dependent on DUSP1. Here it is shown that inhibition of IL-6 expression at the protein level is entirely dependent on DUSP1. The same experiments confirm that dex inhibits expression of IL-12p40 protein independently of DUSP1, emphasizing that both DUSP1-dependent and -independent mechanisms contribute to the anti-inflammatory action of GCs (Abraham *et al.*, 2006). Finally, inhibitory effects of dex on *COX-2* mRNA were largely DUSP1-dependent as previously shown.

In terms of its anti-inflammatory effects, Cpd1 generally behaved like a version of dex with lower efficacy but similar potency. It inhibited IL-6 less effectively than dex but again in an entirely DUSP1-dependent manner. Its inhibitory effects on *COX-2* mRNA were less strong than those of dex but also largely dependent on DUSP1. In all of these cases, inhibitory effects were similar at 10^{-8} and 10^{-6} M. Cpd1-mediated inhibition of TNF expression was also clearly dependent on DUSP1, although in this case the difference between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages became significant only at 10^{-6} M. IL-12p40 expression was equally inhibited by Cpd1 in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages. These observations suggest that, like dex, Cpd1 exerts anti-inflammatory effects in part via DUSP1.

Cpd1 and Cpd2 had similar inhibitory effects on pro-inflammatory genes at 10^{-6} M concentration in wild-type macrophages. At 10^{-8} M concentration, Cpd2 was invariably less effective than Cpd1. Nevertheless, some of the anti-inflammatory effects of Cpd2 were clearly dependent on DUSP1. For example, expression of IL-6 was significantly decreased by either 10^{-8} or 10^{-6} M Cpd2 in *Dusp1*$^{+/+}$ macrophages but not in *Dusp1*$^{-/-}$ macrophages. In the cases of TNF protein or COX-2 mRNA, responses to Cpd2 significantly differed between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages at only one of the two concentrations tested. Inhibition of IL-12p40 expression was independent of DUSP1 as expected. The overall picture is of a compound that depends upon DUSP1 for some of its anti-inflammatory effects but induces DUSP1 expression with relatively low potency.

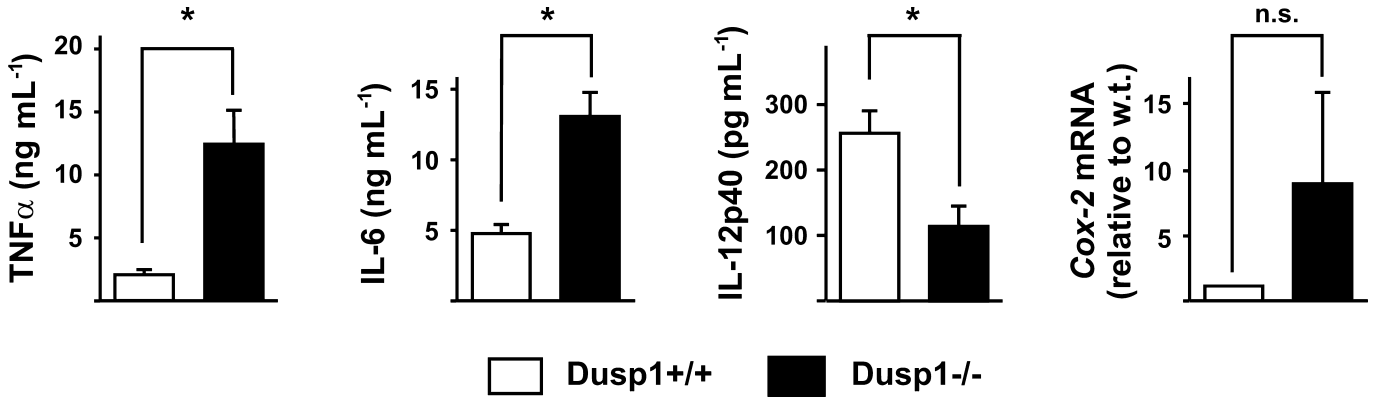

Figure 7

Altered expression of inflammatory mediators in *Dusp1*$^{-/-}$ macrophages. *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ BMM were stimulated with 10 ng·mL^{-1} LPS for 4 h, supernatants were harvested and mRNA was isolated. TNFα and IL-6 proteins were quantified by Luminex, IL-12p40 by ELISA, and *COX-2* mRNA by quantitative PCR. Graphs represent averages from four independent experiments ± SEM. * indicates statistically significant difference between *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ BMM ($P < 0.05$). n.s., not statistically significant.

Discussion

Novel compounds that preferentially mediate transrepression have been predicted to cause fewer side effects than classical GCs (Newton and Holden, 2007; Schacke *et al.*, 2007; Berlin, 2010; De Bosscher *et al.*, 2010). The notional mechanistic uncoupling of therapeutic and harmful consequences of GR activation suggested a straightforward course of action. Safer GR ligands might be discovered through screening strategies based on constructs that contain multimerized binding sites for GR itself (as a reporter for transactivating function) or for NF-κB or AP-1 (as reporters for transrepressing function). However, this idea has its roots in relatively simplistic and now outdated conceptions of how GR controls gene expression. Transcriptional activation by GR is now known to be a remarkably diverse process. Binding sites for GR can extensively vary from the idealized consensus AGAACAnnnTGTTCT, only five or six positions within this sequence being strongly constrained (So *et al.*, 2007; Reddy *et al.*, 2009; John *et al.*, 2011). Single nucleotide variations in binding site sequence can have profound effects on the conformation adopted by GR and the downstream consequences (Meijsing *et al.*, 2009). Only about 0.4% of possible binding sites are recognized by GR in one cell type, this repertoire being dictated by cell type-specific modulation of chromatin accessibility (John *et al.*, 2011). GR also co-operates with a large number of other transcription factors to control transcription (Clark, 2007; Kassel and Herrlich, 2007). Some of these accessory factors are likely to be required for the establishment of domains of open chromatin structure within which GR can bind to DNA. At individual GC-regulated genes, and probably at individual *cis*-acting elements of one gene, GR displays different requirements for transcriptional cofactors (Chen *et al.*, 2006; Galliher-Beckley *et al.*, 2008; John *et al.*, 2011). Finally, GR is extensively post-translationally modified, and GC-responsive elements may display differential requirements for different GR modifications (Beck *et al.*, 2009; Galliher-Beckley and Cidlowski, 2009).

To stand as representative of all GR-mediated transcriptional activation events is therefore an unreasonably large burden for one highly simplified reporter, or even for one or two endogenous genes (Clark, 2007). Some of the problems of extrapolating from simple reporters are well illustrated by the present study. Even within one cell type (A549), the dose-dependence of induction of Dusp1 gene expression by the two SGRMs did not resemble the dose dependence of activation of the GRE reporter (compare Figures 2A and 4A). The predictive value of the reporter became even poorer when other cell types were considered (Figure 4B–D). Individual response elements of the Dusp1 locus were not necessarily better predictors of the behaviour of the endogenous gene. For example, a previous study identified a powerfully GC-responsive region located 4.6 kb upstream of the Dusp1 transcription start site (Tchen *et al.*, 2010). In HeLa cells, this element was quite weakly activated by Cpd1 and Cpd2 compared with dex (Figure 3), whereas the endogenous gene was similarly activated by all three GR ligands at the relevant dose of 10^{-8} M (Figure 4B). At least some of this variation in response of reporter constructs and endogenous GC-regulated genes is probably explained by differential cofactor requirements and variable expression of cofactors in different cell types.

It is hard to escape the conclusion that idealized reporters containing tandem GR binding sites are of little practical help when trying to determine the transactivating properties (and hence dissociated nature) of GR ligands. RU24858, an earlier example of a supposedly dissociated GR ligand, was later found to be capable of up-regulating a subset of GR-regulated genes (Chivers *et al.*, 2006; Janka-Junttila *et al.*, 2006). LGD-5552, a near-relative of Cpd2, was also found to be capable of transcriptional activation but differed from the classical GC prednisolone in the profile of genes activated (Lopez *et al.*, 2008). When a compound is described as being dissociated or as having poor capacity to activate transcription, it should therefore be asked exactly what this means and how it has been demonstrated. It is unclear whether genuine and consistent separation between transactivation and transrepres-

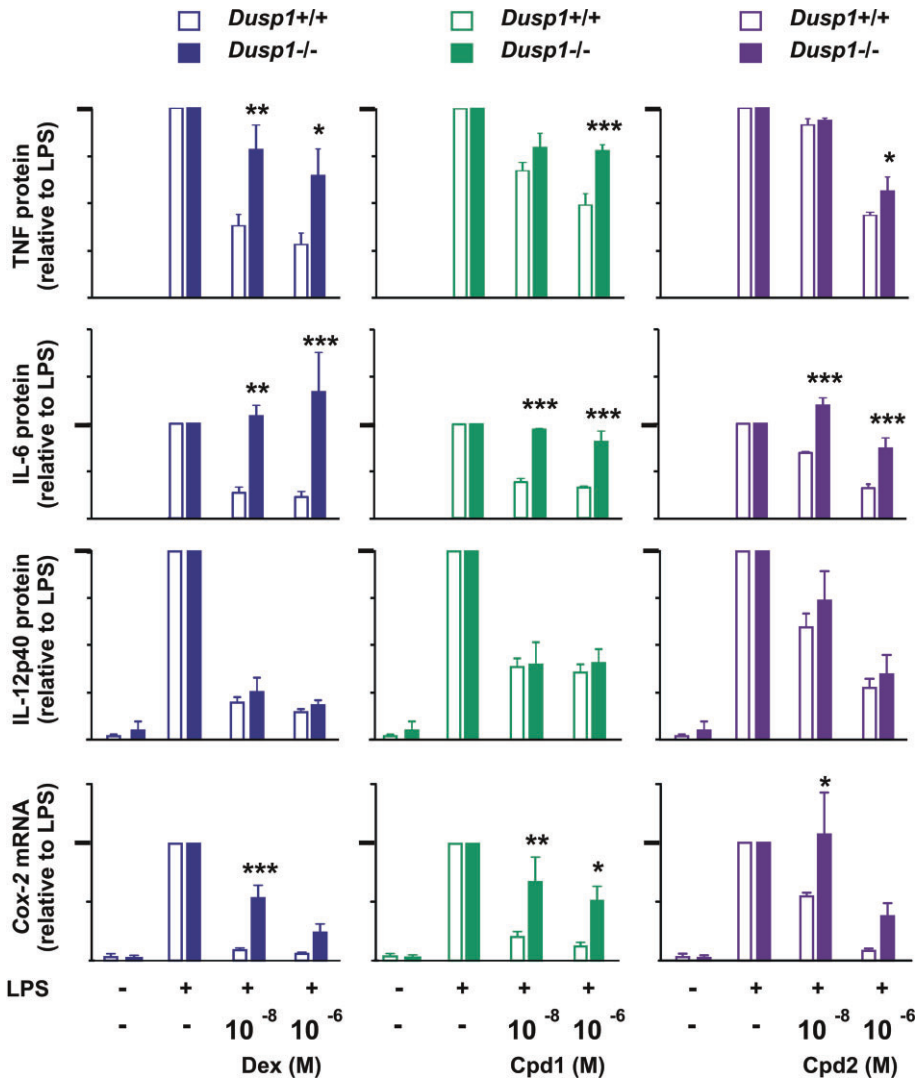

Figure 8

Effects of Cpd1 and Cpd2 on expression of pro-inflammatory mediators in *Dusp1*$^{+/+}$ and *Dusp1*$^{-/-}$ macrophages. BMM from *Dusp1*$^{+/+}$ mice and *Dusp1*$^{-/-}$ mice were pretreated for 2 h with vehicle (0.1% DMSO), 10^{-8} or 10^{-6} M dex, Cpd1 or Cpd2, then challenged with 10 ng·mL^{-1} of LPS for another 4 h. TNF, IL-6 and IL-12p40 protein expression were measured by Luminex or ELISA and *COX-2* mRNA by real-time PCR. Results are presented as percentages of the response in cells treated with LPS and vehicle. The *y*-axes differ in scale. In each case, the 100% level is represented by a heavy tick mark. Graphs indicate mean ± SEM from at least three independent experiments. Statistical analysis is shown for *Dusp1*$^{-/-}$ versus *Dusp1*$^{+/+}$ under the same conditions. ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$.

sion properties of GR can be demonstrated using simple reporters, or whether it can be achieved. We and other investigators have questioned whether such separation is even desirable, given that GCs up-regulate a number of anti-inflammatory factors, and depend on these factors for at least some of their anti-inflammatory effects (Smoak and Cidlowski, 2006; Clark, 2007; Newton and Holden, 2007).

Both Cpd1 and Cpd2 have emerged from deliberate efforts to selectively promote the transrepression rather than transactivation function of GR. With respect to transcriptional activation of GRE reporters and endogenous Dusp1 genes, both compounds behaved like partial agonists of GR. However, Cpd1 did not significantly block transcriptional activation by the full agonist dex (Figure 2D). This surprising finding is reminiscent of the first generation SGRM RU24858, which was also found not to inhibit transcriptional activation by the full agonist dex (Vayssiere *et al.*, 1997). A possible conclusion is that dex and Cpd1 recognize different surfaces of GR and do not bind the receptor in a mutually exclusive manner. This appears unlikely because of the ability of RU486 to antagonize transcriptional activation by either compound (Figure 2C). A second possibility is that Cpd1 has relatively low affinity for GR in intact cells and is therefore not an effective competitor for binding. It should also be pointed out that demonstration of the expected partial antagonism is quite challenging in the case of Cpd1. At the respective concentrations of 10^{-7} and 10^{-6} M, dex and Cpd1 differ by only about 40% in transactivation of the GRE reporter (Figure 2D). This is a relatively small window in which to

demonstrate competitive inhibition by Cpd1 of the response to dex. We cannot conclude that partial antagonism does not occur, only that we have been unable to demonstrate it. Cpd2 being a weaker activator of transcription, partial antagonistic behaviour was more straightforward to demonstrate (Figure 2D). To fully understand the partial agonist/antagonist properties of the two SGRMs requires biochemical and crystallographic studies that are beyond the scope of the present study. In any case, this issue does not effect our major conclusions.

Most importantly, the anti-inflammatory effects of the SGRMs were in direct proportion to their capacity to induce DUSP1 expression in a number of cell types and demonstrably dependent on DUSP1 in mouse macrophages. Cpd2, which had the higher dissociation indices, was the weaker inducer of DUSP1 and the poorer anti-inflammatory agent in all experimental settings used. The importance of DUSP1 as a mediator effect of GR was particularly clearly demonstrated in the case of IL-6. Neither dex, Cpd1 nor Cpd2 was capable of decreasing expression of IL-6 protein in *Dusp1*$^{-/-}$ macrophages. It is ironic that IL-6 is a well-characterized NF-κB target, whose inhibition by GCs has usually been interpreted in terms of transrepression.

Cpd1 is closely related to ZK 245186 (otherwise known as BOL-303242-X or Mapracorat), which is being tested as a novel drug for dermatological or opthalmological indications such as atopic dermatitis, dry-eye syndrome and postoperative eye inflammation (Schacke *et al.*, 2009; Zhang *et al.*, 2009; Cavet *et al.*, 2010; Pfeffer *et al.*, 2010; Shafiee *et al.*, 2010). ZK 245186 was found to inhibit JNK and p38 MAPK phosphorylation in corneal epithelial cells subjected to hyperosmolar stress (Cavet *et al.*, 2010). Inhibition of the MAPKs is thought to contribute to the therapeutic effect of the SGRM and may be mediated by up-regulation of DUSP1. The classical GC dex induces expression of Dusp1 in primary human lens epithelium (Gupta *et al.*, 2005). In the present study, we were not able to assess responses to ZK 245186 itself, and we acknowledge the danger of extrapolating too far from *in vitro* studies. Nevertheless, we consider it quite possible that the anti-inflammatory efficacy and safety of SGRMs like ZK 245186 have little to do with whether or not these compounds are 'dissociated'. This raises important questions about how the properties of the current generation of SGRMs should be interpreted and how the next generation of SGRMs might best be identified.

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Conflicts of interest

The authors have no conflict of interest.

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